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Special Review Series – Gene Manipulation and Integrative Physiology

Embryonic stem cells and gene targeting

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The development of gene targeting technology, the exchange of an endogenous allele of a target gene for a mutated copy via homologous recombination, and the application of this technique to murine embryonic stem cells has made it possible to alter the germ-line of mice in a predetermined way. Gene targeting has enabled researchers to generate mouse strains with defined mutations in their genome allowing the analysis of gene function *in vivo*. This review presents the essential tools and methodologies used for gene targeting that have been developed over the past decade. Special emphasis has been laid on the available embryonic stem cell lines and the importance of the genetic background. Also, the state-of-the art of gene targeting approaches in species other than mice will be discussed. *Experimental Physiology* (2000) **85.6, 603–613.**

Embryonic stem (ES) cells and the application of gene targeting technology in ES cells have had a profound impact on genetic analysis in the mouse during the past decade.

Nearly two decades ago two independent groups described the isolation of undifferentiated embryonic stem cells from explanted mouse pre-implantation embryos (Evans & Kaufman, 1981; Martin, 1981). In 1984 Bradley *et al.* demonstrated the capacity of ES cells to colonize the germ-line of chimeric mice upon injection into host embryos. The following year, the so-called gene targeting procedure, the exchange of an endogenous allele of a target gene for a transfected mutated copy via homologous recombination events, was first achieved in the β -globin gene in cultured mammalian cells by Smithies *et al.* (1985). This approach was then successfully applied in embryonic stem cells with the selectable *Hprt*-locus by Thomas & Capecchi (1987) and Doetschman *et al.* (1988). In 1989 followed the first publication of an HPRT-knock-out mouse, an animal model for the human Lesh Nyhan syndrome, generated with HPRT-deficient ES cells (Koller *et al.* 1989). In the same year the first mouse carrying a targeted inactivation of a non-selectable gene, the proto-oncogene *c-Abl*, was reported (Schwartzberg *et al.* 1989). Since then the number of published targeted mouse mutants has risen dramatically (for review see Brandon *et al.* 1995). The technology has been further improved with the development of inducible trans-activator or recombinase systems that enable the temporal and spatial control of gene modifications thereby further expanding the flexibility of gene targeting. At present, the zoo of 'designer' mutant mice ranges from animals carrying constitutive knock-out of genes, subtle mutations,

gene replacements, inversions, deletions, chromosomal translocations and tissue-specifically inducible genes.

This review describes the essential tools and methodologies used for gene targeting that have been developed over the past decade. The first part is focussed on embryonic stem cells: the ES cell lines currently available for gene targeting, the importance of the genetic background and mutant phenotype and ES cells as vehicles for generating transgenic mice.

The second part includes: the basic techniques used to generate genetically modified mouse models and the state-of-the-art of gene targeting approaches in species other than mice.

Embryonic stem cells

ES cell lines that keep their pluripotency after transfection and selection procedures are essential for the introduction of selected targeted mutations into the germ-line of mice. Pluripotent ES cells are established *in vitro* from the inner cell mass of explanted blastocyst-stage embryos (Evans & Kaufman, 1981; Martin, 1981; Ledermann, 1997). ES cells are maintained in a pluripotent state by co-culturing with mitotically inactivated feeder cells, such as embryonic fibroblasts, and/or the addition of a differentiation-inhibiting activity called leukaemia inhibitory factor (LIF, Smith & Hooper, 1987; Williams *et al.* 1988). The developmental potential of ES cells may be investigated *in vitro* by omitting LIF from the culture medium, or *in vivo* by microinjection of ES cells into morulae or into the blastocoel cavity of a blastocyst. When pluripotent the ES cells participate in normal development of the embryo and contribute to all three germ

Table 1. Mouse substrain 129 -derived ES cell lines used in gene targeting experiments

Cell line	Gene	Reference
D3 (129/SvPas)	$\alpha 5$ integrin	Yang <i>et al.</i> (1993)
	Fibronectin	George <i>et al.</i> (1993)
CCE (129/SvEv)	<i>c-abl</i>	Schwartzberg <i>et al.</i> (1989)
	Neurofibromatosis type-1	Brannan <i>et al.</i> (1994)
AB-1 (129/SvEvBrd)	wnt-1	McMahon & Bradley (1990)
	ICAM-1	Sligh <i>et al.</i> (1993)
J1 (129/SvJae)	Neurotrophin-3	Ernfors <i>et al.</i> (1994)
	p75 ^{NGFR}	Lee <i>et al.</i> (1992)
R1* (129/SvJ)	Engrailed-1	Wurst <i>et al.</i> (1994)
E14TG2a † (129/OlaHsd)	IL-4	Kuehn <i>et al.</i> (1991)
	TGF- α	Luetke <i>et al.</i> (1993)
HM-1 † (129/OlaHsd)	ERCC-1	McWhir <i>et al.</i> (1993)
TC-1 (129/Sv)	Mpl/G-CSFR	Stoffel <i>et al.</i> (1999)

* This cell line can be used for the generation of aggregation chimeras. † These cell lines are HPRT deficient and can be used both for positive (HAT-medium) and negative (6-TG-medium) selection (see Fig. 1).

Table 2. C57BL/6-, BALB/c-, DBA/1- and MRL-derived ES cell lines used for gene targeting

Cell line	Gene	Reference
Bruce 4 (C57BL/6)	MHC class II Aa	Koentgen <i>et al.</i> (1993)
	CD3 ζ/η	Malissen <i>et al.</i> (1993)
BL/6-III (C57BL/6)	Ig κ	Zou <i>et al.</i> (1993)
	Perforin	Kägi <i>et al.</i> (1994)
	CD23	Yu <i>et al.</i> (1994)
	PBGD	Lindberg <i>et al.</i> (1996)
	IL-5	Kopf <i>et al.</i> (1996)
BALB/c-I	IL-4	Noben-Trauth <i>et al.</i> (1996)
	IL-4R α	Mohrs <i>et al.</i> (1999)
DBA-252 (DBA/1)	FLAP	Roach <i>et al.</i> (1995)
MRL	Ep2	Goulet <i>et al.</i> (1997)

layers including the germ-line of the resulting chimeric animal (Evans & Kaufman, 1981). Alternatively, to form chimeras, ES cells can simply be aggregated with host morulae (Wood *et al.* 1993a,b), thus omitting the technically intricate micro-injection step.

The derivation of germ-line competent ES cell lines from species other than mice has so far not been possible (see also Outlook). Even in the mouse the currently used strategies revealed differences in the efficiencies of ES cell establishment when using various inbred strains (author's unpublished observation). And, thus far, using a standard protocol our laboratory has not been able to derive stable ES cell lines from NOD (non-obese diabetes) and Friend virus B mouse strains. This certainly reflects the poor knowledge about the biology of ES cells. Only recently, elements of the intracellular signal transduction pathways regulating stem cell renewal have been identified. It has been demonstrated that pluripotency of ES cells is maintained through the action of cytokines that engage the gp130 cytokine receptor-subunit (Niwa, 1998; Matsuda, 1999).

Mouse substrain 129-derived embryonic stem cell lines

The vast majority of germ-line competent ES cell lines used in gene targeting experiments have been derived from blastocysts of sublines of the mouse substrain 129 (Table 1). Historically the 129/Sv strain was chosen because this strain is characterized by a high incidence of spontaneous testicular teratomas or teratocarcinomas and initially served as a source of embryonal carcinoma cell lines (Silver *et al.* 1983).

Inbred strain-derived ES cell lines

In the beginning, the derivation of ES cells from mouse strains other than 129 seemed not to be possible (Smith, 1992). However, after modifying the original protocol used for the establishment of 129-derived ES cells, embryonic stem cells from other so-called non-permissive inbred mouse strains have been successfully isolated (Ledermann & Bürki, 1991; Kawase *et al.* 1994; Roach *et al.* 1995; McWhir *et al.* 1996; Brook & Gardner, 1997; Noben-Trauth *et al.* 1996).

The successful use of inbred strain-derived ES cells for gene targeting experiments depends largely on optimal culture

conditions, especially serum-, feeder cell- and water quality, and the technical skill of the investigator. For example, C57BL/6-derived ES cell lines easily lose their ability to colonize the germ-line upon injection into a host blastocyst in contrast to 129-derived and BALB/c ES cells (author's unpublished observations).

Mutant phenotype and genetic background

The analysis of targeted mutations revealed that an observed mutant phenotype is often less severe than expected, and/or detectable only in a subset of the tissues which normally express the target gene. This has been observed in a variety of knock-out mice. The explanation for this observation is the existence of functional redundancy between genes (Gridley, 1991; Doherty, 1993; Strohmman, 1994) and compensatory mechanisms between gene family members, an example being the myogenic factors, see below. A possibility for investigating the redundancy and compensatory mechanisms is the generation of double (or, if necessary, multiple) knock-out animals. This has been demonstrated for a variety of gene families (for review see Müller, 1999) including two members of the *MyoD* family of myogenic transcriptional regulators: *MyoD* and *Myf-5*. Single knock-out mice for these genes develop fairly normal amounts of muscle (Rudnicki *et al.* 1992; Braun *et al.* 1992) whereas in double-knock-out animals no muscle is formed (Rudnicki *et al.* 1993). In contrast, *Il-2* and *Il-4* double knock-out animals have not exhibited any cumulative phenotype, indicating functional independence of IL-2 and IL-4 (Sadlack *et al.* 1994).

Another important finding with knock-out mice is the fact that the genetic background of the induced mutation affects the phenotype of the homozygous mutant mice (for review see Gerlai, 1996; Doetschman, 1999; Müller, 1999). One of the first examples was reported by Ramirez-Solis *et al.* (1993). They demonstrated incomplete penetrance and variable expressivity of a *Hoxb-4* mutation in a 129SvEv/C57BL/6j hybrid background compared with complete penetrance of the induced mutation in an inbred 129SvEv background. This is not surprising because with the establishment of mouse inbred strains it has become clear that the genetic background plays an important role in the susceptibility of mice to many disorders. These effects might be due to polymorphic modifier genes in different mouse strains. Even mouse 129 substrains from which the majority of ES cell lines have been established and used for the generation of gene targeted mice show extensive genetic variability (Simpson *et al.* 1997) and differ in reproductivity and behaviour (Festing, 1996).

The general strategy for generating gene targeted mice so far, includes the use of 129-derived ES cell lines and the backcrossing of the induced mutation onto other inbred strain backgrounds such as C57BL/6 or BALB/c. The mutation can then be backcrossed onto different inbred strain backgrounds to generate congenic strains and to identify modifier loci. There may be also benefits to looking at mixed strain backgrounds. Mixed genetic background knock-out mice often have a wider range of phenotypes (Doetschman, 1999).

The traditional approach requires backcrossing for about 10 generations in order to reach a 99 % inbred strain background. This process takes about 2–3 years. The number of necessary generations may be limited by using the speed congenics approach, a marker-assisted breeding (Markel *et al.* 1997). However, even with extensive backcrossing the DNA immediately surrounding the targeted locus would not be of inbred strain origin. Genes closely linked to the disrupted locus could influence the phenotype of the induced mutation which complicates the interpretation of the results. With the availability of inbred strain-derived ES cell lines as listed in Table 2, it is now possible to induce a mutation on the genetic inbred background of choice without laborious and time-consuming breeding. This is especially important for the availability of control animals when behavioural studies have to be carried out.

Generation of transgenic mice via ES cells

The generation of transgenic mice via the ES cell route, a technique first described by Gossler *et al.* (1986) and Robertson *et al.* (1986) has not found widespread application since the generation of transgenic animals via pronuclear microinjection is much faster and less laborious. However, the ES cell route to transgenesis offers some advantages compared with the classical microinjection technique.

Inbred strain-derived ES cells may be used to generate transgenic mice since the efficiency for producing transgenic mice on these backgrounds via pronuclear microinjection is very low (author's unpublished observations) due to the overall reduced reproductive efficiency in inbred strain backgrounds (Brinster *et al.* 1985). For example, the efficient generation of transgenic BALB/c mice has been demonstrated by Dinkel *et al.* (1999) when using BALB/c embryonic stem cells. The transfected ES cell clones can be screened *in vitro* for correct configuration and/or copy number of the transgene and expression, cells with a desired genetic alteration can be chosen for injection into blastocysts. The transgenic ES cells can also be analysed regarding their *in vitro* differentiation potential.

Embryonic stem cells can be used for the targeted insertion (knock-in) of transgenic sequences thereby overcoming the problem of unreliable transgene expression (for review see Jasin *et al.* 1996). The foreign sequences may be placed under control of the regulatory elements of that locus. The transgene sequences may be inserted, leaving the target locus functionally intact or, alternatively, they may replace part of the target gene, thereby disrupting the endogenous gene. An endogenous murine gene may thus be replaced by a reporter gene, e.g. the *Escherichia coli lacZ* gene. Mansour *et al.* (1990) have introduced the *lacZ* gene into the murine *Int-2* locus so that the endogenous promoter of the targeted gene controls the reporter gene. The expression of these genes can be followed *in situ* throughout embryogenesis of the mutant animal. Homozygous embryos will allow the visual assessment at the cellular level of gene inactivation effects in transgenic mice. An elegant way to study the potential role of cytokines

and their receptors in hematopoietic cell fate decisions has been published by Stoffel *et al.* (1999). They replaced the thrombopoietin receptor gene (*Mpl*) with a chimeric construct encoding the extracellular domain of *Mpl* and the cytoplasmic domain of the granulocyte colony-stimulating factor (*Gcsfr*). The chimeric receptor binds thrombopoietin but signals through the *Gcsfr* intracellular domain. Using this approach it was demonstrated that the cytoplasmic domain of *Gcsfr* can functionally replace *Mpl* signalling *in vivo* to support normal megakaryopoiesis and platelet formation.

Knock-in approaches may also be used to drive the tissue-specific expression of the Cre recombinase in tissue-specific gene targeting approaches (see also Tissue-specific gene targeting). For example, Rickert *et al.* (1997) have achieved B-cell specific expression when targeting the *cre* gene into the *Cd19* locus.

An endogenous murine gene of choice can also be replaced by human sequences (Stacey *et al.* 1994; Bonaventure *et al.* 1999). This concept of replacing the endogenous murine gene by human sequences via gene targeting or via the combination of knock-out mice with mice overexpressing human transgenes will become increasingly important for the *in vivo* testing of drug efficacy.

Gene targeting

Before starting a gene targeting experiment the researcher has to carefully plan the experimental strategy of the targeting approach and should consider both options for constitutive and conditional gene targeting and the genetic background of the induced mutation. An optimal strategy is dependent on the biological problem to be analysed and therefore no general

strategy can be recommended. The appropriate design of a targeting construct and the choice of a suitable ES cell line will result in mouse models with higher predictive values and limit the time and expense associated with the mouse breeding.

The equipment and experimental procedures used to generate gene-targeted mice are accurately described in laboratory handbooks by Hogan *et al.* (1986), Wassarman & De Pamphilis (1993), Torres & Kühn (1997), Kopf (1997) and Joyner (1998).

Types of targeting constructs

Two types of constructs have been used to modify target genes in ES cells: replacement-type vectors and insertion-type vectors (Fig. 1). Replacement-type vectors contain part of the genomic target sequence with a mutant foreign sequence such as a positive selection cassette, inserted within the coding region. The vector is linearized outside the region of homology. Such vectors recombine with the target gene by a double cross-over event, resulting in the replacement of the chromosomal DNA by the targeting construct.

Insertion-type vectors are linearized within the region of homology. Such vectors are inserted entirely into the target gene locus by a single crossover event, leading to a partial duplication of the homologous DNA. The position of the linearization site will affect the structure of the recombinant allele. Therefore, for the design of an insertion-type vector it is important to consider the potential RNA species and splicing possibilities of the recombinant allele. With this type of vector the target gene can either be inactivated or mutated through the introduction of subtle mutations.

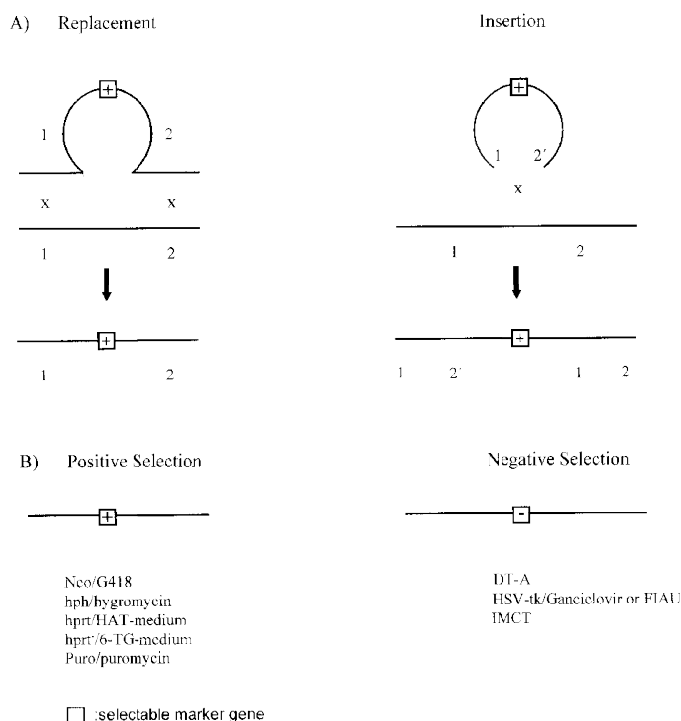


Figure 1. Types of targeting constructs

The selection cassettes should, whenever possible, be removed after identification of homologous recombination events. It has been shown that strong promoters driving the selection marker expression may interfere with the expression of neighbouring genes (Fiering *et al.* 1995). Removal of the selection cassette is easily achieved by applying the Cre/*loxP* recombinase system (Fig. 2, see also Tissue-specific gene targeting).

Introduction of subtle mutations

Several strategies have been designed to introduce subtle mutations (e.g. point mutations) into the target locus without leaving selectable sequences in the target gene, to analyse gene function and for correction of specific gene defects in gene therapy.

These strategies include the 'hit and run' or 'in and out' strategy that requires two steps of recombinations and is based on an insertion-type vector containing a subtle mutation in the target gene and a positive and a negative selection marker in the plasmid backbone (Hasty *et al.* 1991; Valancius & Smithies, 1991). The 'tag and exchange' strategy involves also two sequential gene targeting steps using two different replacement type vectors (Askew *et al.* 1993). A modification of this protocol is the so-called 'plug and socket' approach developed by Detloff *et al.* (1994). A much simpler method for introducing subtle mutations into the target gene has been developed by Reid *et al.* (1991) and Davis *et al.* (1992). The selection marker and the target construct containing minor alterations are located on separate DNA fragments and introduced simultaneously into ES cells by co-electroporation. ES cell clones with a homologous recombination event in the target locus together with a random insertion of the selection marker have been identified. The selection marker will be separated from the targeted allele during meiosis following transmission through the germ-line.

The most commonly applied method for introducing subtle mutations is based on the site specific recombinases from bacteriophage P1 or yeast. The 38 kDa Cre recombinase from bacteriophage P1, which has been shown to be more efficient in ES cells than the yeast recombinase FLP (for review see Rossant & McMahon, 1999), catalyses a *loxP*-dependent site-specific recombination in both prokaryotic and eukaryotic cells (Fig. 2). In the first step, a sequence flanked by two 34 bp *loxP* sites in the same orientation can be inserted into a target

locus. In the second step, it is efficiently removed from the chromosome by subsequent expression of the Cre recombinase. For the introduction of subtle mutations, for example, the selection marker flanked by two *loxP* sites is inserted into an intron of the mutated genomic targeting sequences. This construct is used to replace part of the target locus by homologous recombination. After positive selection, the Cre enzyme is transiently expressed in the targeted ES cells clones. Cre catalyses a *loxP*-dependent recombination thereby excising the potentially disruptive selectable marker gene, leaving a single *loxP* site in the intron of the mutated target gene. Alternatively, for removal of the selection cassette, animals carrying the *loxP*-flanked selection marker are being bred with so-called Cre-deleter mice (see also Tissue-specific gene targeting).

Recently, a FLP recombinase variant with improved activity, FLPe, has been reported (Rodriguez *et al.* 2000) as an alternative to, or in conjunction with, the Cre-*loxP* system.

Enrichment for gene targeting events

Electroporation is the transfection method of choice for the introduction of a targeting construct into ES cells. However, the disadvantage of this technically very simple method is the low transformation efficiency (10^{-3}) and therefore it is necessary to include a positive selection marker (Fig. 1). The most commonly used selection cassette is the prokaryotic neomycin phosphotransferase (*neo*) gene driven by the phosphoglycerate kinase-1 (*Pgk-1*) promoter. Other selection markers commonly used in gene targeting experiments are the prokaryotic hygromycin B phosphotransferase- (*hph*) and the puromycin (*puro*) gene. Alternatively, the use of *Hprt* minigenes as the selectable marker in HPRT-deficient ES cells has been described (Reid *et al.* 1990; Selfridge *et al.* 1992).

Homologous recombination occurs approximately 1000-fold less frequently than non-homologous recombination, therefore methods have been developed to enrich for homologous recombination events. A widely applied method includes the use of a positive and a negative selection marker and does not require the expression of the target gene in ES cells (Mansour *et al.* 1988). The targeting construct is based on a replacement-type vector containing the positive selection marker within the region of homology and the negative selection marker at one or both ends of the homologous DNA. Genes for the negative

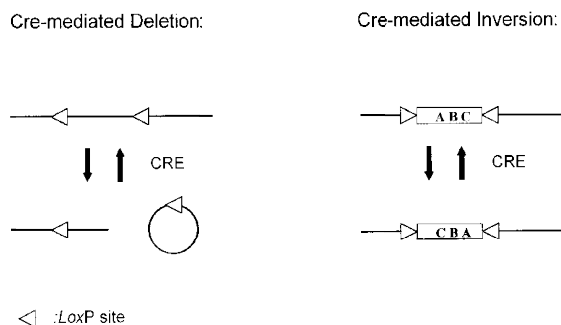


Figure 2. The Cre/*loxP* recombination system

selection that have been used in this approach include the herpes simplex virus thymidine kinase gene which converts nucleoside analogues, such as ganciclovir or 1(1-2-deoxy-2-fluoro- β -D-arabinofuransosyl)-5-iodouracil (FIAU) into toxic metabolites, the diphtheria toxin A gene (Yagi *et al.* 1990) and, more recently, immunotoxin-mediated negative selection has been applied (Kobayashi *et al.* 1996). In the first step, the positive selection is applied for cells that have integrated the targeting construct in their genome. In the second step, the negative selection is applied against cells with a random insertion of the construct, since cells carrying a targeted integration event must have lost the terminal negative selection marker. Positive-negative selection enhances the ratio of targeted to non-targeted events only by 5- to 10-fold due to occasional loss of the negative selection marker during random integration.

The enrichment for homologous recombination events can be increased by using a positive selection marker lacking 5' (promoter) or 3' (polyadenylation signal) regulatory sequences, provided the target gene is expressed in ES cells (reviewed by Bradley *et al.* 1992). Another strategy for enrichment of homologous recombination events was demonstrated by Lindberg *et al.* 1996. In this approach, the artificial splice acceptor site was placed in front of the coding sequence of a promoterless *neo*^R gene and this cassette was inserted into the first intron of the target gene. As expected, the insertion resulted in the splicing of the first exon to the *neo*^R coding sequence, thereby competing with the normal splicing, and the generation of a fusion protein.

Double knock-out of target genes

The two alleles of the target gene can be inactivated consecutively in ES cells through two rounds of homologous recombination. This can either be obtained by using two constructs containing different positive selection markers (Te Riele *et al.* 1990; Mortensen *et al.* 1991), or by increasing the G418 concentration after the first round of homologous recombination (Mortensen *et al.* 1992), thereby selecting for cells with the selectable marker also inserted in the second allele. Using this approach, gene function may be studied directly in ES cells *in vitro* or in a chimeric *in vivo* situation, or in the RAG-2 blastocyst complementation assay for lymphocyte development (see also Tissue-specific gene targeting).

Screening for targeted ES cell clones

Two types of screening procedures are used to identify ES cell clones carrying a targeted integration of the construct DNA: polymerase chain reaction (PCR) and Southern blot analysis (Southern, 1975). Both methods rely upon the specific juxtaposition of vector components and target locus sequences after homologous recombination. Asymmetric construct

The PCR is the most sensitive method for identifying cells with a targeted integration event (Frohman & Martin, 1990). Two oligonucleotide primers are used to amplify a specific fragment created by the homologous recombination event. One primer is complementary to sequences unique to the

target locus and the other is unique to sequences within the targeting construct. PCR amplification of the expected fragment is possible only when these primers are correctly juxtaposed by a homologous recombination event.

Southern blot analysis is usually used to confirm the PCR results. With the choice of restriction digest and probes for hybridization, the wild-type allele can readily be distinguished from the targeted allele since predicted novel restriction fragments are generated by the homologous recombination event.

Parameters affecting the frequency of homologous recombination

The major parameters influencing the frequency of homologous recombination in ES cells include the target locus itself or the locus region, the length of homologous DNA used in the targeting construct, transcriptional activity of the target locus, the penetrance of the selectable marker (reviewed by Frohman & Martin, 1989; Fung-Leung & Mak, 1992) and the use of isogenic DNA (Van Deursen & Wieringa, 1992; Te Riele *et al.* 1992).

Conditional gene targeting

The constitutive inactivation of genes in the mouse leads to mice that are constantly deficient for the product of the deleted gene. This may cause embryonic lethality, if the gene product is crucial for development. Its function in later stages of development cannot be studied in this case. Also, a constitutively deficient animal may compensate for the loss of a gene product. The resulting phenotype may be close to the wild-type animal, although the gene product has an important function. These problems of a constitutive knock-out of a gene can be avoided by tissue-specific and inducible gene disruption strategies.

Tissue-specific gene targeting

Cells with a double knock-out of a particular gene may be studied in a chimeric situation. In this respect, an elegant method, named RAG-2-deficient blastocyst complementation, to study the role of a target gene product in T- and B-lymphocyte development, has been described by Chen *et al.* (1993). Injection of normal ES cells into RAG-2-deficient blastocysts will lead to the development of a normal population of exclusively ES-derived lymphocytes in the chimeric animal, since RAG-2-deficient mice lack mature B- and T-lymphocytes. Using ES cells with homozygous mutations, RAG-2-deficient blastocyst complementation could provide a physiological assay to directly determine the potential role of almost any gene in the development and/or function of lymphocytes in the chimeric animal.

The most widely adopted approach to achieve tissue-specific gene targeting is the use of the Cre-*loxP* system. The gene encoding the site-specific recombinase Cre is introduced as a regulated transgene driven by a tissue-specific promoter. As transgene expression is usually not fully penetrant, this approach may be problematic for cells and organs with a considerable regeneration capacity, such as bone marrow or

the liver, because non-deleted cells most likely will displace the transgenic cells. Alternatively, the *cre* gene is inserted by 'knock-in' gene targeting behind a suitable promoter an example being the *Cd19* locus for B-cell specific expression (Rickert *et al.* 1997; see also Generation of transgenic mice via ES cells). The Cre-transgenic mice are then combined with gene-targeted mice carrying *loxP* flanked-genomic regions of the target gene to be deleted or modified. The target gene remains expressed until the *cre* gene is induced. Cre then catalyses site-specific recombination between the *loxP* sites, deleting the sequences inbetween and thereby leading to gene silencing.

The temporal and spatial patterns of Cre activity may be analysed by crossing the Cre-expressing mice with so-called 'reporter-mice'. These mice carry a *loxP*-flanked stop codon between an ubiquitous promoter and, for example, the *lacZ*-reporter gene (Mao *et al.* 1999; Soriano, 1999). In all tissues expressing Cre the stop codon will be removed and the Cre activity can be monitored by X-Gal staining.

The generation of Cre transgenic mice using different tissue-specific promoters revealed that Cre was sometimes unexpectedly expressed already in the early embryo prior to germ cell development. This resulted in the so-called 'deleter mice' that upon combination with *loxP* flanked target mice give rise to offspring carrying the deleted gene in every tissue including the germ-line. These mice however, are an elegant alternative to the Cre-mediated recombination in ES cells and may be used for example, when combined with gene-targeted mice, to remove the *loxP*-flanked selection cassette.

The following databases provide a source of information for those Cre transgenic mice already in existence: A. Nagy, private database: <http://www.mshri.on.ca/develop/Nagy/Cre.htm>; European mouse mutant archive (EMMA): <http://www.emma.rm.cnr.it>; The Jackson Laboratory, Bar Harbor: <http://www.jax.org>.

Inducible gene targeting

With the development of conditional gene targeting it is now possible to control gene targeting both in a tissue-specific and temporal manner. Several inducible systems have been developed to achieve inducible expression of Cre controlled either at the transcriptional or at the post-transcriptional level (reviewed by Gingrich & Roder, 1998; Rossant & McMahon, 1999). However, all inducible systems developed so far still have disadvantages such as background activity in the absence of the inducer, efficiency of Cre-mediated recombination and toxic effects caused by the inducer

Transcriptional control-based approaches. The first inducible tissue-specific gene deletion was reported in 1995 by Kühn *et al.* The *cre* gene was expressed under the control of an inducible promoter of the mouse *Mx1* gene. This transgenic mouse was combined with a mouse line carrying a *loxP* flanked DNA polymerase β gene as a target. By treating the double transgenic mice with IFN- α or double-stranded RNA, both agents that induce the *Mx1* promoter, gene deletion was induced. The deletion occurred within 2 days after injection of

the inducers and the degree of deletion varied from up to 100 % in the liver to 10 % in brain. This inducible system may have a drawback for inducible gene inactivations in the immune system since the inducing agents have severe side-effects.

Gossen & Bujard (1992) have adapted tetracycline-responsive promoter elements from bacterial genes to mammalian gene control. The gene for the tetracycline-repressor protein has been fused to the gene of a viral activator protein. This hybrid activator can bind to a target gene that carries the operator sequence recognized by the tetracycline repressor. The viral activator will turn the gene on. When tetracycline is present, however, it will bind to the hybrid protein and prevent its binding to the operator. The target gene is therefore shut down. The reverse system has also been applied. A mutant tetracycline repressor that requires tetracycline derivatives for specific DNA binding was fused to the viral transactivator. By giving the drug derivative, the target gene can be turned on (Gossen *et al.* 1995).

Another system for switching genes on or off has been applied by No *et al.* (1996) by using the *Drosophila* steroid hormone ecdysone. Ecdysone binds to a receptor, consisting of a heterodimer, which then moves into the nucleus, binds to its recognition sequence and activates genes. Since normal mouse genes do not respond to the insect hormone, ecdysone induction can be used in the mouse *in vivo*.

Post-transcriptional control-based approaches. Several groups have applied inducible systems based on the activity of fusion proteins between the mutated ligand binding domain (LBD) of a steroid receptor and Cre recombinase. The fusion transcript is driven by a tissue-specific promoter.

Cre fusion proteins with the LBD's of the human progesterone receptor (PG) and the human estrogen receptor (ER) have been generated. Cre activity is induced by administration of synthetic but not natural ligands and hormone-inducible Cre-mediated recombination has been demonstrated *in vitro* in ES cells (Kellendonk *et al.* 1996; Zhang *et al.* 1996) and also in transgenic mice (Feil *et al.* 1996; Vasioukhin *et al.* 1999). The current limitations of these systems, e.g. the oestrogen-receptor system, are the high levels of inducer (4-hydroxy-tamoxifen, OHT) needed to activate the Cre fusion because of the low-binding affinity of the mutated binding domain. Recently, Indra *et al.* (1999) have reported an improved system that show a 10-fold higher sensitivity to OHT induction.

Outlook

The application of gene targeting technology in murine embryonic stem cells over the past decade has enabled researchers to generate tailor-made 'designer mutant mice'. The precise genetic alterations of the mouse germ-line range from subtle mutations, gene replacements, inversions, deletions, chromosome rearrangements to tissue-specific inducible gene targeting allowing temporal and spatial control. The tissue-specific and inducible approaches will be further improved and in the near future it will be possible to reversibly switch on and off any gene in any tissue at any chosen point in time.

So far, gene targeting technology has been restricted to mice as germ-line competent ES cells have been established for this species only. ES cells that morphologically resemble murine ES cells have been isolated from the golden hamster (Doetschman *et al.* 1988), pig (Wheeler *et al.* 1994), sheep (Notiarianni *et al.* 1991), cow (Evans *et al.* 1990), primates (Thomson & Marshall, 1998) and humans (Thomson *et al.* 1998). Some of these cell lines were capable of producing chimeric animals but failed to colonize the germ-line.

The recently developed nuclear transfer technology, the generation of genetically identical animals (clones) via the transfer of nuclei from cultured, differentiated cells into enucleated, unfertilized eggs, will probably circumvent the problem of isolating stable ES cell lines from other species (Wilmut *et al.* 1997; Schnieke *et al.* 1997; Cibelli *et al.* 1998; Baguisi, 1999). The application of gene targeting approaches in somatic cells followed by nuclear transfer will enable the application of genetic engineering to any species as it is now performed in the mouse (Suraokar & Bradley, 2000; McCreath *et al.* 2000). Furthermore, nuclear transfer technology will lead to improved commercial livestock, e.g. prion protein-deficient cattle and sheep in order to overcome the problems associated with bovine spongiform encephalitis (BSE) and scrapie. Also, this methodology will be applied for human therapeutic cloning: embryonic stem cells from patients will be derived by nuclear transfer techniques. These ES cells may then be differentiated *in vitro* in order to derive genetically matched cells and tissues for transplantation (Lanza *et al.* 1999).

In the future, the limitation will not be the tools for genome manipulation in every species but the time and expense associated with generating and maintaining large numbers of mutant animals, as well as ethical considerations. Therefore, it will become much more important to thoroughly plan the strategy for genome engineering and to optimize methods for phenotypic analysis that do not require maintaining large numbers of mutant animals.

ASKEW, G. R., DOETSCHMAN, T. & LINGREL, J. B. (1993). Site-directed point mutations in embryonic stem cells: a gene-targeting tag-and-exchange strategy. *Molecular and Cellular Biology* **13**, 4115–4124.

BAGUISI, A. (1999). Production of goats by somatic cell nuclear transfer. *Nature Biotechnology* **17**, 456–461.

BEDELL, M. A., LARGAESPADA, D. A., JENKINS, N. A. & COPELAND, N. G. (1997). Mouse models of human disease. Part II: Recent progress and future directions. *Genes and Development* **11**, 11–43.

BONAVENTURE, P., UMANS, L., BAKKER, M. H., CRAS, P., LANGLOIS, X., LUYTEN, W. H., MEGENS, A. A., SERNEELS, L., VAN LEUVEN, F. & LEYSEN, J. E. (1999). Humanization of mouse 5-hydroxytryptamine1B receptor gene by homologous recombination: *in vitro* and *in vivo* characterization. *Molecular Pharmacology* **56**, 54–67.

BRADLEY, A., EVANS, M., KAUFMAN, M. H. & ROBERTSON, E. (1984). Formation of germ-line chimeras from embryo-derived teratocarcinoma cell lines. *Nature* **309**, 255–257.

BRADLEY, A., HASTY, P., DAVIS, A. & RAMIREZ-SOLIS, R. (1992). Modifying the mouse: design and desire. *BioTechnology* **10**, 534–539.

BRANDON, E. P., IDZERDA, R. L. & MCNIGHT, G. S. (1995). Targeting the mouse genome: a compendium of knockouts. *Current Biology* **5**, 625–634 (Part I), 758–765 (Part II), 873–881 (Part III).

BRANNAN, C. I., PERKINS, A. S., VOGEL, K. S., RATNER, N., NORDLUND, M. L., REID, S. W., BUCHBERG, A. M., JENKINS, N. A., PARADA, L. F. & COPELAND, N. G. (1994). Targeted disruption of the neurofibromatosis type-1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. *Genes and Development* **8**, 1019–1029.

BRAUN, T., RUDNICKI, M. A., ARNOLD, H. H. & JAENISCH, R. (1992). Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell*, **71**, 369–382.

BRINSTER, R. L., CHEN, H. Y., TRUMBauer, M. E., YAGEL, M. K. & PALMITER, R. D. (1985). Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proceedings of the National Academy of Sciences of the USA* **82**, 4438.

BROOK, F. A. & GARDNER, R. L. (1997). The origin and efficient derivation of embryonic stem cells in the mouse. *Proceedings of the National Academy of Sciences of the USA* **94**, 5709–5712.

CHEN, J., GORMAN, J. R., STEWART, B., WILLIAMS, B., JACKS, T. & ALT, F. W. (1993). RAG-2-deficient blastocyst complementation: an assay of gene function in lymphocyte development. *Proceedings of the National Academy of Sciences of the USA* **90**, 4528–4532.

CIBELLI, J. B., STICE, S. L., GOLUEKE, P. J., KANE, J. J., JERRY, J., BLACKWELL, C., PONCE DE LEON, F. A. & ROBL, J. M. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280**, 1256–1258.

DAVIS, A. C., WIMS, M. & BRADLEY, A. (1992). Investigation of coelectroporation as a method for introducing small mutations into embryonic stem cells. *Molecular and Cellular Biology* **12**, 2769–2776.

DETLOFF, P. J., LEWIS, J., JOHN, S. W., SHEHEE, W. R., LANGENBACH, R., MAEDA, N. & SMITHIES, O. (1994). Deletion and replacement of the mouse adult beta-globin genes by a 'plug and socket' repeated targeting strategy. *Molecular and Cellular Biology* **14**, 6936–6943.

DINKEL, A., AICHER, W. K., WARNATZ, K., BÜRKI, K., EIBEL, H. & LEDERMANN, B. (1999). Efficient generation of transgenic BALB/c mice using BALB/c embryonic stem cells. *Journal of Immunological Methods* **223**, 255–260.

DOETSCHMAN, T. (1999). Interpretation of phenotype in genetically engineered mice. *Laboratory Animal Science* **49**, 137–143.

DOETSCHMAN, T., MAEDA, N. & SMITHIES, O. (1988). Targeted mutation of the *hprt* gene in mouse embryonic stem cells. *Proceedings of the National Academy of Sciences of the USA* **85**, 8583–8587.

DOHERTY, P. C. (1993). Virus infection in mice with targeted gene disruptions. *Current Opinion in Immunology* **5**, 479–483.

ERNFORS, P., LEE, K. F., KUCERA, J. & JAENISCH, R. (1994). Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell* **77**, 503–512.

EVANS, M. J. & KAUFMANN, M. H. (1981). Establishment in culture of pluripotent cells from mouse embryos. *Nature* **292**, 154–156.

EVANS, M. J., NOTARIANNI, E., LAURIE, S. & MOOR, R. M. (1990). Maintenance and differentiation in culture of pluripotential embryonic cell lines from pig blastocysts. *Theriogenology* **33**, 879–901.

FEIL, R., BROCARD, J., MASCREZ, B., LEMEUR, M., METZGER, D. & CHAMBON, P. (1996). Ligand-activated site-specific recombination in mice. *Proceedings of the National Academy of Sciences of the USA* **93**, 10887–10890.

- FESTING, M. (1996). Inbred strains of mice. *Mouse Genome* **94**, 523–677.
- FIERING, S., EPNER, E., ROBINSON, K., ZHUANG, Y., TELLING, A., HU, M., MARTIN, D. I., ENVER, T., LEY, T. J. & GROUDINE, M. (1995). Targeted deletion of 5'HS2 of the murine beta-globin LCR reveals that it is not essential for proper regulation of the beta-globin locus. *Genes and Development* **9**, 2203–2213.
- FROHMAN, M. A. & MARTIN, G. R. (1989). Cut, paste, and save: new approaches to altering specific genes in mice. *Cell* **56**, 145–147.
- FROHMAN, M. A. & MARTIN, G. R. (1990). In *PCR Protocols: A Guide to Methods and Applications*, ed. INNIS, M. A., GELFAND, D. H., SNINSKY, J. J. & WHITE, T. J., pp. 228–236. Academic Press, San Diego.
- FUNG-LEUNG, W. P. & MAK, T. W. (1992). Embryonic stem cells and homologous recombination. *Current Opinion in Immunology* **4**, 189–194.
- GEORGE, E. L., GEORGES-LABOUESSE, E. N., PATEL-KING, R. S., RAYBURN, H. & HYNES, R. O. (1993). Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development* **119**, 1079–1091.
- GERLAI, R. (1996). Gene-targeting studies of mammalian behavior: is it the mutation of the background phenotype? *Trends in Neurosciences* **19**, 177–181.
- GINGRICH, J. R. & RODER, J. (1998). Inducible gene expression in the nervous system of transgenic mice. *Annual Review of Neuroscience* **21**, 377–405.
- GOSSEN, M. & BUJARD, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences of the USA* **89**, 5547–5551.
- GOSSEN, M., FREUDSIEB, S., BENDER, G., MÜLLER, G., HILLEN, W. & BUJARD, H. (1995). Transcriptional activation by tetracycline in mammalian cells. *Science* **268**, 1766–1769.
- GOSSLER, A., DOETSCHMAN, T., KORN, R., SERFLING, E. & KEMLER, R. (1986). Transgenesis by means of blastocyst-derived embryonic stem cell lines. *Proceedings of the National Academy of Sciences of the USA* **83**, 9065.
- GOULET, J. L., WANG, C.-Y. & KOLLER, B. H. (1997). Embryonic stem cell lines from MRL mice allow genetic modification in a murine model of autoimmune disease. *Journal of Immunology* **159**, 4376–4381.
- GRIDLEY, T. (1991). Insertional versus targeted transgenesis. *New Biologist* **3**, 1025–1034.
- HASTY, P., RAMIREZ-SOLIS, R., KRUMLAUF, R. & BRADLEY, A. (1991). Introduction of a subtle mutation into the Hox-2.6 locus in embryonic stem cells. *Nature* **350**, 243–246.
- HOGAN, B., CONSTANTINI, F. & LACY, E. (1986). *Manipulating the Mouse Embryo. A Laboratory Manual*. Cold Spring Harbor Laboratory, NY, USA.
- INDRA, A. K., WAROT, X., BROCARD, J., BORNERT, J.-M., XIAO, J.-H., CHAMBON, P. & METZGER, D. (1999). Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. *Nucleic Acids Research* **27**, 4324–4327.
- JASIN, M., MOYNAHAN, M. E. & RICHARDSON, C. (1996). Targeted transgenesis. *Proceedings of the National Academy of Sciences of the USA* **93**, 8804–8808.
- JOYNER, A. L. (ed) (1998). *Gene Targeting. A Practical Approach*. IRL Press, Oxford.
- KÄGI, D., LEDERMANN, B., BÜRKI, K., SEILER, P., ODERMATT, B., OLSEN, K. J., PODACK, E. R., ZINKERNAGEL, R. M. & HENGARTNER, H. (1994). Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* **369**, 31–37.
- KAWASE, E., SUEMORI, H., TAKAHASHI, N., OKAZAKI, K., HASHIMOTO, K. & NAKATSUJI, N. (1994). Strain difference in establishment of mouse embryonic stem (ES) cell lines. *International Journal of Developmental Biology* **38**, 385–390.
- KOBAYASHI, K., OHYE, T., PASTAN, I. & NAGATSU, T. (1996). A novel strategy for the negative selection in mouse embryonic stem cells operated with immunotoxin-mediated cell targeting. *Nucleic Acids Research* **24**, 3653–3655.
- KOENTGEN, F., SÜSS, G., STEWART, C., STEINMETZ, M. & BLUETHMANN, H. (1993). Targeted disruption of the MHC class II Aa gene in C57BL/6 mice. *International Immunology* **5**, 957–964.
- KELLENDONK, C., TRONCHE, F., MONAGHAN, A. P., ANGRAND, P. O., STEWART, F. & SCHULTZ, G. (1996). Regulation of Cre recombinase activity by the synthetic steroid RU 486. *Nucleic Acids Research* **24**, 1404–1411.
- KOLLER, B. H., HAGEMANN, L. J., DOETSCHMAN, T., HAGAMAN, J. R., HUANG, S., WILLIAMS, P. J., FIRST, N. L., MAEDA, N. & SMITHIES, O. (1989). Germ-line transmission of a planned alteration made in a hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cells. *Proceedings of the National Academy of Science of the USA* **86**, 8927–8931.
- KOPF, M. (1997). In *Immunological Methods Manual*, vol. 1, pp. 165–182.
- KOPF, M., BROMBACHER, F., HODGKIN, P. D., RAMSAY, A. J., MILBOURNE, E. A., DAI, W. J., OVINGTON, K. S., BEHM, C. A., KÖHLER, G., YOUNG, I. G. & MATTHAEI, K. I. (1996). IL-5-deficient mice have a developmental defect in CD5⁺ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. *Immunity* **4**, 15–24.
- KÜHN, R., RAJEWSKY, K. & MÜLLER, W. (1991). Generation and analysis of interleukin-4 deficient mice. *Science* **254**, 707–710.
- KÜHN, R., SCHWENK, F., AGUET, M. & RAJEWSKY, K. (1995). Inducible gene targeting in mice. *Science* **269**, 1427–1429.
- LANZA, R. P., CIBELLI, J. B. & WEST, M. D. (1999). Human therapeutic cloning. *Nature Medicine* **5**, 975–977.
- LEDERMANN, B. (1997). Establishment of embryonic stem cell lines. In *Immunological Methods Manual*, vol. 1, 165–182.
- LEDERMANN, B. & BÜRKI, K. (1991). Establishment of a germ-line competent C57BL/6 embryonic stem cell line. *Experimental Cell Research* **197**, 254–258.
- LEE, K. F., LI, E., HUBER, L. J., LANDIS, S. C., SHARPE, A. H., CHAO, M. V. & JAENISCH, R. (1992). Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. *Cell* **69**, 737–749.
- LINDBERG, R. L. P., PORCHER, C., GRANDCHAMP, B., LEDERMANN, B., BÜRKI, K., BRANDNER, S., AGUZZI, A. & MEYER, U. A. (1996). Porphobilinogen deaminase deficiency in mice causes a neuropathy resembling that of human hepatic porphyria. *Nature Genetics* **12**, 195–199.
- LUETTEKE, N. C., QIU, T. H., PEIFFER, R. L., OLIVER, P., SMITHIES, O. & LEE, D. C. (1993). TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. *Cell* **73**, 263–278.
- MCGREATH, K. J., HOWCROFT, J., CAMPBELL, K. H. S., COLMAN, A., SCHNIEKE, A. E. & KIND, A. J. (2000). Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. *Nature* **405**, 1066–1069.
- MCMAHON, A. P. & BRADLEY, A. (1990). The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62**, 1073–1085.

- MCWHIR, J., SCHNIEKE, A. E., ANSELL, R., WALLACE, H., COLMAN, A., SCOTT, A. R. & KIND, A. J. (1996). Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a non-permissive genetic background. *Nature Genetics* **14**, 223–226.
- MALISSEN, M., GILLET, A., ROCHA, B., TRUCY, J., VIVIER, E., BOYER, C., KÖNTGEN, F., BRUN, N., MAZZA, G., SPANOPOULOU, E., GUY-GRAND, D. & MALISSEN, B. (1993). T cell development in mice lacking the CD3-zeta/eta gene. *EMBO Journal* **12**, 4347–4355.
- MCWHIR, J., SELFRIDGE, J., HARRISON, D. J., SQUIRES, S. & MELTON, D. W. (1993). Mice with DNA repair gene (ERCC-1) deficiency have elevated levels of p53, liver nuclear abnormalities and die before weaning. *Nature Genetics* **5**, 217–224.
- MANSOUR, S. L., THOMAS, K. R. & CAPECCHI, M. R. (1988). Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* **336**, 348–352.
- MAO, X., FUJIWARA, Y. & ORKIN, S. (1999). Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. *Proceedings of the National Academy of Sciences of the USA* **96**, 5037–5042.
- MARKEL, P., SHU, P., EBELING, C., CARLSON, G. A., NAGLE, D. L., SMUTKO, J. S. & MOORE, K. J. (1997). Theoretical and empirical issues for marker-assisted breeding of congenic mouse strains. *Nature Genetics* **17**, 280–284.
- MARTIN, G. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proceedings of the National Academy of Sciences of the USA* **78**, 7634–7638.
- MATSUDA, T., NAKAMURA, T., NAKAO, K., ARAI, T., KATSUKI, M., HEIKE, T. & YOKOTA, T. (1999). STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO Journal* **18**, 4261–4269.
- MOHRS, M., LEDERMANN, B., KÖHLER, G., DORFMÜLLER, A., GESSNER, A. & BROMBACHER, F. (1999). Differences between IL-4 and IL-4 receptor α -deficient mice in chronic Leishmaniasis reveal a protective role for IL-13 Receptor signalling. *Journal of Immunology* **162**, 7302–7308.
- MORTENSEN, R. M., CONNER, D. A., CHAO, S., GEISTERFER-LOWRANCE, A. A. T. & SEIDMAN, J. G. (1992). Production of homozygous mutant ES cells with a single targeting construct. *Molecular and Cellular Biology* **12**, 2391–2395.
- MORTENSEN, R. M., ZUBIAUR, M., NEER, E. J. & SEIDMAN, J. G. (1991). Embryonic stem cells lacking a functional inhibitory G-protein subunit (α i2) produced by gene targeting of both alleles. *Proceedings of the National Academy of Sciences of the USA* **88**, 7036–7040.
- MÜLLER, U. (1999). Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis. *Mechanisms of Development* **82**, 3–21.
- NIWA, H., BURDON, T., CHAMBERS, I. & SMITH, A. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes and Development* **12**, 2048–2060.
- NO, D., TSO-PANG, Y. & EVANS, R. M. (1996). Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proceedings of the National Academy of Sciences of the USA* **93**, 3346–3351.
- NOBEN-TRAUTH, N., KOHLER, G., BÜRKI, K. & LEDERMANN, B. (1996). Efficient targeting of the IL-4 gene in a BALB/c embryonic stem cell line. *Transgenic Research* **5**, 487–491.
- NOTARIANNI, E., GALLI, C., LAURIE, S., MOOR, R. M. & EVANS, M. J. (1991). Derivation of pluripotent, embryonic cell lines from the pig and sheep. *Journal of Reproduction and Fertility* **43**, 255–260.
- RAMIREZ-SOLIS, R., ZHENG, H., WHITING, J., KRUMLAUF, R. & BRADLEY, A. (1993). Hoxb-4 (Hox-2.6) mutant mice show homeotic transformation of a cervical vertebra and defects in the closure of the sternal rudiments. *Cell* **73**, 279–294.
- REID, L. H., GREGG, R. G., SMITHIES, O. & KOLLER, B. H. (1990). Regulatory elements in the introns of the human HPRT gene are necessary for its expression in embryonic stem cells. *Proceedings of the National Academy of Sciences of the USA* **87**, 4299–4303.
- REID, L. H., SHESLEY, E. G., KIM, H. S. & SMITHIES, O. (1991). Cotransformation and gene targeting in mouse embryonic stem cells. *Molecular and Cellular Biology* **11**, 2769–2777.
- RICKERT, R. C., ROES, J. & RAJEWSKY, K. (1997). B lymphocyte-specific, Cre-mediated mutagenesis in mice. *Nucleic Acids Research* **25**, 1317–1378.
- ROACH, M., STOCK, J. L., BYRUM, R., KOLLER, B. H. & MCNEISH, J. D. (1995). A new embryonic stem cell line from DBA/1lacJ mice allows genetic modification in a murine model of human inflammation. *Experimental Cell Research* **221**, 520–525.
- ROBERTSON, E., BRADLEY, A., KUEHN, M. & EVANS, M. (1986). Germline transmission of genes introduced into cultured pluripotent cells by retroviral vector. *Nature* **323**, 445.
- RODRIGUEZ, C. I., BUCHHOLZ, F., GALLOWAY, J., SEQUERRA, R., KASPER, J., AYALA, R., STEWART, F. A. & DYMECKI, S. M. (2000). High-efficiency deleter mice show that FLP ϵ is an alternative to Cre-loxP. *Nature Genetics* **25**, 139–140.
- ROSSANT, J. & MCMAHON, A. (1999). Creating mouse mutants – a meeting review on conditional mouse genetics. *Genes and Development* **13**, 142–145.
- RUDNICKI, M. A., BRAUN, T., HINUMA, S. & JAENISCH, R. (1992). Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell* **71**, 383–390.
- RUDNICKI, SCHNEGELSBERG, P. N., STEAD, R. H., BRAUN, T. & ARNOLD, H. H. & JAENISCH, R. (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* **75**, 1351–1359.
- SADLACK, B., KUHN, R., SCHORLE, H., RAJEWSKY, K., MULLER, W. & HORAK, I. (1994). Development and proliferation of lymphocytes in mice deficient for both interleukins-2 and -4. *European Journal of Immunology* **24**, 281–281.
- SCHNIEKE, A. E., KIND, A. J., RITCHIE, W. A., MYCOCK, K., SCOTT, A. R., RITCHIE, M., WILMUT, I., COLMAN, A. & CAMPBELL, K. H. (1997). Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science* **278**, 2130–2133.
- SCHWARTZBERG, P. L., GOFF, S. P. & ROBERTSON, E. J. (1989). Germ-line transmission of a c-abl mutation produced by targeted gene disruption in ES cells. *Science* **246**, 799–803.
- SELFIDGE, J., POW, A. M., MCWHIR, J., MAGIN, T. M. & MELTON, D. W. (1992). Gene targeting using a mouse HPRT minigene/HPRT-deficient embryonic stem cell system: inactivation of the mouse ERCC-1 gene. *Somatic Cell and Molecular Genetics* **18**, 325–336.
- SILVER, L. M., MARTIN, G. R. & STRICKLAND, S. (Eds.) (1983). *Teratocarcinoma Stem Cells*, pp. 690. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.
- SIMPSON, E. M., LINDER, C. C., SARGENT, E. E., DAVISSON, M. T., MOBRAATEN, L. E. & SHARP, J. J. (1997). Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. *Nature Genetics* **16**, 19–27.
- SLIGH, J. E., BALLANTYNE, C. M., RICH, S. S., HAWKINS, H. K., SMITH, C. W., BRADLEY, A. & BEAUDET, A. L. (1993). Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. *Proceedings of the National Academy of Sciences of the USA* **90**, 8529–8533.

- SMITH, A. G. (1992). Mouse embryo stem cells: their identification, propagation and manipulation. *Seminars in Cell Biology* **3**, 385–399.
- SMITH, A. G. & HOOPER, M. L. (1987). Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. *Developmental Biology* **121**, 1–9.
- SMITHIES, O., GREGG, R. G., BOGGS, S. S., KORALEWSKI, M. A. & KUCHERLAPATI, R. S. (1985). Insertion of DNA sequences into the human chromosomal β -globin locus by homologous recombination. *Nature* **317**, 230–234.
- SORIANO, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nature Genetics* **21**, 70–71.
- SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.
- STACEY, A., SCHNIEKE, A., MCWHIR, J., COOPER, J., COLMAN, A. & MELTON D. W. (1994). Use of double-replacement gene targeting to replace the murine alpha-lactalbumin gene with its human counterpart in embryonic stem cells and mice. *Molecular and Cellular Biology* **14**, 1009–1016.
- STOFFEL, R., ZIEGLER, S., GHILARDI, N., LEDERMANN, B., DE SAUVAGE, F. J. & SKODA, R. C. (1999). Permissive role of thrombopoietin and granulocyte colony-stimulating factor receptors in hematopoietic cell fate decisions *in vivo*. *Proceedings of the National Academy of Sciences of the USA* **96**, 698–702.
- STROHMAN, R. (1994). Epigenesis: the missing beat in biotechnology? *Bio/Technology* **12**, 156–164.
- SURAOKAR, M. & BRADLEY, A. (2000). Targeting sheep. *Nature* **405**, 1004–1005.
- TE RIELE, H., MAANDAG, E. R. & BERNIS, A. (1992). Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. *Proceedings of the National Academy of Sciences of the USA* **89**, 5128–5132.
- TE RIELE, H., MAANDAG, E. R., CLARKE, A., HOOPER, M. & BERNIS, A. (1990). Consecutive inactivation of both alleles of the pim^{-1} proto-oncogene by homologous recombination in embryonic stem cells. *Nature* **348**, 649–651.
- THOMAS, K. & CAPECCHI, M. (1987). Site-directed mutagenesis by gene targeting in mouse embryonic stem cells. *Cell* **51**, 503–512.
- THOMSON, J. A., ITSKOVITZ-ELDOR, J., SHAPIRO, S. S., WAKNITZ, M. A., SWEIRGIEL, J. J., MARSHALL, V. S. & JONES, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147.
- THOMSON, J. A. & MARSHALL, V. S. (1998). Primate embryonic stem cells. *Current Topics in Developmental Biology* **38**, 133–165.
- TORRES, R. M. & KÜHN, R. (1997). *Laboratory Protocols for Conditional Gene Targeting*. Oxford University Press, Oxford.
- VALANCIUS, V. & SMITHIES, O. (1991). Testing an 'in-out' targeting procedure for making subtle genomic modifications in mouse embryonic stem cells. *Molecular and Cellular Biology* **11**, 1402–1408.
- VAN DEURSEN, J. & WIERINGA, B. (1992). Targeting of the creatine kinase M gene in embryonic stem cells using isogenic and nonisogenic vectors. *Nucleic Acids Research* **20**, 3815–3820.
- VASIOUKHIN, V., DEGENSTEIN, L., WISE, B. & FUCHS, E. (1999). The magical touch: Genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. *Proceedings of the National Academy of Sciences of the USA* **96**, 8551–8556.
- WASSARMAN, P. M. & DE PAMPHILIS, M. L. (1993). In *Guide to Techniques in Mouse Development*. Academic Press, New York.
- WHEELER, M. B. (1994). Development and validation of swine embryonic stem cells. *Reproduction, Fertility and Development* **6**, 563–568.
- WILLIAMS, R. L., HILTON, D. J., PEASE, S., WILLSON, T. A., STEWART, C. L., GEARING, D. P., WAGNER, E. F., METCALF, D., NICOLA, N. A. & GOUGH, N. M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* **336**, 684–687.
- WILMUT, I., SCHIEKE, A. E., MCWHIR, J., KIND, A. J. & CAMPBELL, K. H. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810–813.
- WOOD, S. A., ALLEN, N. D., ROSSANT, J., AUERBACH, A. & NAGY, A. (1993a). Non-injection methods for the production of embryonic stem cell-embryo chimaeras. *Nature* **365**, 87–89.
- WOOD, S. A., PASCOE, W. S., SCHMIDT, C., KEMLER, R., EVANS, M. J. & ALLEN, N. D. (1993b). Simple and efficient production of embryonic stem cell-embryo chimeras by coculture. *Proceedings of the National Academy of Sciences of the USA* **90**, 4582–4585.
- WURST, W., AUERBACH, A. B. & JOYNER, A. L. (1994). Multiple developmental defects in Engrailed-1 mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum. *Development* **120**, 2065–2075.
- YAGI, T., IKAWA, Y., YOSHIDA, K., SHIGETANI, Y., TAKEDA, N., MABUCHI, L., YAMAMOTO, T. & AIZAWA, S. (1990). Homologous recombination at c-fyn locus of mouse embryonic stem cells with use of diptheria toxin A-fragment gene in negative selection. *Proceedings of the National Academy of Sciences of the USA* **87**, 9918–9992.
- YANG, J. T., RAYBURN, H. & HYNES, R. O. (1993). Embryonic mesodermal defects in alpha 5 integrin-deficient mice. *Development* **119**, 1093–1105.
- YU, P., KOSCO-VILBOIS, M., RICHARDS, M., KÖHLER, G. & LAMERS, M. C. (1994). Negative feedback regulation of IgE synthesis by murine CD23. *Nature* **369**, 753–756.
- ZHANG, Y., RIESTERER, C., AYRALL, A. M., SABLITZKY, F., LITTLEWOOD, T. D. & RETH, M. (1996). Inducible site-directed recombination in mouse embryonic stem cells. *Nucleic Acids Research* **24**, 543–548.
- ZOU, Y. R., TAKEDA, S. & RAJEWSKY, K. (1993). Gene targeting in the Ig kappa locus: efficient generation of lambda chain-expressing B cells, independent of gene rearrangements in Ig kappa. *EMBO Journal* **12**, 811–820.

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